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Microvessel Count Predicts Metastasis and Prognosis in Patients With Gastric Cancer

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Background and Objectives: In order to evaluate the clinical relevance of angiogenesis in patients with gastric cancer, we investigated the microvessel count in gastric cancer tissues and compared the results with several clinicopathologic factors and prognosis.
Methods: A total of 55 patients with gastric cancer were included in this study. Microvessel count was determined by immunohistochemical staining using antifactor VIII-related antigen antibody. Histologically recognizable blood vessels within tissue sections served as internal control for immunostaining. Normal mouse IgG diluted to an equivalent protein concentration was used as a negative control in place of the primary antibody in each experiment.
Result: The microvessel count ranged from 4.4 to 39.8 and the median count was 15.6. Microvessel count was significantly higher in patients with hepatic metastasis, lymph node metastasis, adjacent organ invasion, and lymphatic invasion. In patients who had undergone a curative operation, survival time in the hypervascular group was significantly shorter than that in the hypovascular group.
Conclusions: Microvessel count correlated well with tumor progression and may serve as a useful prognostic factor in patients with gastric cancer.
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KEY WORDS: tumor angiogenesis; antifactor VIII-related antigen antibody; gastric cancer

INTRODUCTION

Tumor angiogenesis is considered to be essential for tumor growth and progression [1]. Recently, new technology for counting these microvessels using antifactor VIII-related antigen antibody, which recognizes endothelial cells, has been developed [2], and several investigators have reported that the microvessel count increases with tumor progression and is a useful prognostic factor in patients with breast cancer [3,4], lung cancer [5], ovarian cancer [6], colon cancer [7], prostate cancer [8], renal cancer [9], melanoma [10], and head and neck cancer [11]. However, little information concerning microvessel density in patients with gastric cancer is available at present [12,13]. In order to determine the clinical relevance of microvessel count in patients with gastric cancer, we measured the microvessel count in a total of 55 primary gastric carcinomas and compared the results with several clinicopathologic factors and prognosis.

MATERIALS AND METHODS

Patients

A total of 55 gastric cancer patients who had undergone gastrectomy at our department from January 1991 to December 1995 were included in this study. Patient characteristics are shown in Table I. Thirty-five patients were male and 20 were female. Median age was 64.0

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TABLE I. Gastric Cancer Microvessel Study: Patient Characteristics

Sex	Male: 35	Female: 20			
Age (yr)	35–82 (median: 64.0)				
Peritoneal metastasis	−	49			
	+	6			
Hepatic metastasis	−	44			
	+	11			
Macroscopic type[c]	0	19			
	1	1			
	2	6			
	3	20			
	4	7			
	5	2			
Depth of tumor invasion[d]	t1	17			
	t2	23			
	t3	10			
	t4	5			
Lymph node metastasis[a]	n0	21			
	n1	13			
	n2	6			
	n3	3			
	n4	12			
Histological type[b]	pap	3			
	tub1	12			
	tub2	18			
	por1	10			
	por2	4			
	sig	5			
	muc	3			
Conclusive stage	Ia	13			
	Ib	10			
	II	8			
	IIIa	2			
	IIIb	2			
	IVb	20			

[a]n0, no evidence of lymph node metastasis; n1, metastasis to group 1 lymph node; n2, metastasis to group 2 lymph node; n3, metastasis to group 3 lymph node; n4, metastasis to group 4 lymph node.

[b]pap, papillary adenocarcinoma; tub1, well-differentiated tubular adenocarcinoma; tub2, moderately differentiated tubular adenocarcinoma; por1, solid type poorly differentiated adenocarcinoma; por2, nonsolid type poorly differentiated adenocarcinoma; sig, signet-ring cell carcinoma; muc, mucinous adenocarcinoma.

[c]0, superficial, flat tumors; 1, polypoid tumors, sharply demarcated from surrounding mucosa; 2, ulcerated carcinomas with sharply demarcated and raised margins; 3, ulcerated carcinoma without definite limits, infiltrating into the surrounding wall; 4, diffusely infiltrating carcinomas; 5, nonclassifiable carcinomas.

[d]t1, tumor invasion of mucosa or submucosa; t2, tumor invasion of muscularis propria or subserosa; t3, tumor penetration of serosa; t4, tumor invasion of adjacent structures.

(range: 35–82) years. Patient staging and clinicopathological characteristics were determined by the Japanese Classification of Gastric Carcinoma [14]. Eleven of the 55 patients had hepatic metastasis, 6 had peritoneal dissemination, and 34 had lymph node metastasis. No patients had received prior chemotherapy or radiotherapy before surgery. Tumor specimens were fixed in 10% formaldehyde and embedded in paraffin. After surgery, all, except for Stage Ia patients, received adjuvant chemotherapy consisting of oral 5-fluorouracil for 2 or 3 years.

Immunohistochemical Techniques

Immunohistochemical studies were performed on formalin-fixed and paraffin-embedded tissues using the avidin-biotin immunoperoxidase complex technique. Sections (4 μm thick) were mounted on glass slides. These sections were dewaxed in xylene, dehydrated in ethanol, then incubated with 3% hydrogen peroxidase for 30 min to block endogenous peroxidase activity. After washing with phosphate-buffered saline (PBS), the sections were incubated in 10% normal rabbit serum for 20 min to reduce nonspecific antibody binding. The sections were then incubated with a 1:100 diluted mouse monoclonal antibody F8/86, which recognizes factor VIII-related antigen (Dakopatts, Glostrup, Denmark), at 4°C overnight. After three washes with PBS, the sections were incubated with 10 μg/ml biotinylated rabbit antimouse IgG (Nichirei Corp., Tokyo, Japan) for 30 min. They were then treated with peroxidase-conjugated streptavidin (Nichirei) for 30 min at a concentration of 100 μg/ml followed by three washes with PBS. Finally, 0.02% diaminobenzidine (Wako Pure Chemical Industries, Osaka, Japan) and 1% hydrogen peroxidase were reacted for 10 min. Histologically recognizable blood vessels within tissue sections served as internal control for immunostaining. Normal mouse IgG diluted to an equivalent protein concentration was used as a negative control in place of the primary antibody in each experiment.

Microvessel Counting

The slides were screened under low magnification to identify the highest vascular density area within the tumor, and five areas of highest microvessel density were selected for examination at 200 times magnification. The average of the five areas was calculated. Brown-stained single cells or cell clusters with or without a vessel lumen structure were counted as microvessels. The count was performed by two investigators (M.A. and K.A.) without the knowledge of other clinicopathologic factors.

Statistical Analyses

The microvessel count according to several clinicopathologic factors was compared and analyzed by Mann-Whitney's U-test. The survival curves were calculated using the Kaplan-Meier method and analyzed by the log-rank test.

RESULTS

Endothelial cells were seen as brown granules by immunohistochemical staining using antifactor VIII-related antigen and were easily distinguishable from the background tissues (Fig. 1). Microvessel count ranged from 4.4 to 39.8 with a mean ± standard deviation (SD) of 16.9 ± 9.0 (median count; 15.6). (Fig. 2). Microvessel counts according to the clinicopathologic factors are shown in Table II. There was no statistically significant difference among the microvessel count and peritoneal metastasis, histological type of the tumor, and venous invasion. However, the microvessel count was significantly higher in patients with hepatic metastasis, lymph node metastasis, adjacent organ invasion (t4), and lymphatic invasion. Concerning macroscopic type, the microvessel count was

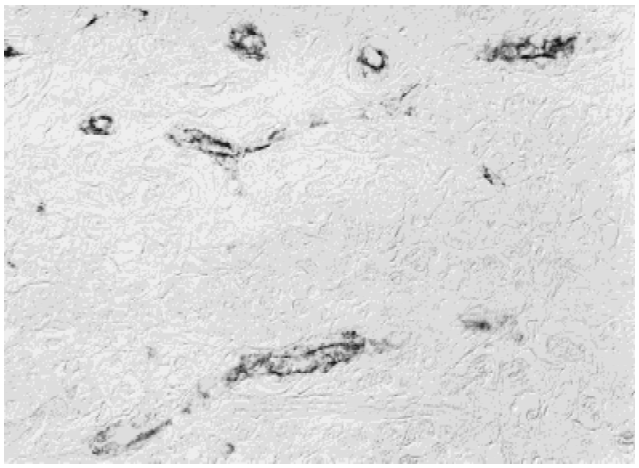

Fig. 1. Immunohistochemical staining of endothelial cells with antifactor VIII-related antigen (×200). Fine granular staining can be observed in endothelial cells.

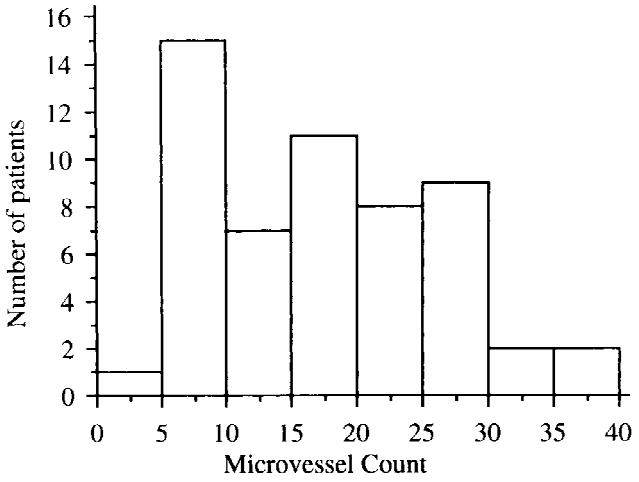

Fig. 2. Distribution of the microvessel count in 55 gastric cancer tissues.

higher in type 3 tumor than in the other tumor types. In spite of the fact that the microvessel count was higher in patients with advanced disease, a statistically significant correlation was not observed between the microvessel count and the conclusive stage of the tumor.

Since the median microvessel count was 15.6, we set the cutoff value at 15.6 and compared the overall survival rate between the hypervascular group (microvessel count $\geq$ 15.6) and the hypovascular group (microvessel count $<$ 15.6). In all patients investigated, the overall survival rate was slightly lower in the hypervascular group, but there were no statistically significant differences between the two groups (Fig. 3A). However, in 35 patients who had undergone curative resection, the survival rate in the hypervascular group was significantly worse than in the hypovascular group ($P < 0.05$) (Fig. 3B). There were no significant differences of age, stage, and adjuvant chemotherapy between the two groups.

DISCUSSION

The significance of tumor angiogenesis has been well recognized in recent years. Angiogenesis is essential to supply nutrients and oxygen to tumor tissues with the growth of the tumor. After a new tumor has attained the size of a few millimeters in diameter, further expansion of the tumor cell population requires the induction of new capillary blood vessels. This angiogenesis is mediated by several growth factors produced by tumor cells or mesenchymal cells. Once the tumor has obtained angiogenic properties, rapid tumor growth, bleeding, and the potential for metastasis are observed [1]. Several investigators have reported a significant correlation with angiogenesis, expressed as the number of microvessels within the tumor, and tumor metastasis and prognosis in a clinical setting [2–13]. The possible mechanism of these findings has been attributed to the structural features of neovascularized vessels. Since the proliferating capillaries have fragmented membranes and are leaky, it is easy for cancer cells to invade neovascularized vessels [15]. The greater the number of tumor vessels, the greater the opportunity for tumor cells to enter the circulation. Bosari et al. [4] reported that an association was not found among microvessel count and tumor size, ploidy status, and the proliferative S-phase fraction of tumors in a study on breast cancer. The only microvessel count association found was with peritumoral vascular invasion, and it was speculated that this process may be correlated with distant metastasis [4]. In the present study, we were unable to distinguish any significant differences with microvessel count in terms of venous invasion; however, the microvessel count was significantly higher in patients with hepatic metastasis. Accordingly, angiogenesis, vascular invasion, and distant metastasis are thought to be biologically related processes in gastric cancer.

The correlation between lymph node metastasis and microvessel density has been reported in several solid tumors such as breast, renal, and lung cancer. With regard to gastric cancer, the association between microvessel density and lymph node metastasis is controversial. Tanigawa et al. [13] found no correlation between microvessel count and lymph node metastasis. Maeda et al. [12] reported that the microvessel count was significantly higher in patients with lymph node metastases than in those without such metastasis. In the present study, we observed a significant association among microvessel count and adjacent organ invasion, lymphatic invasion, and lymph node metastasis, prompting us to speculate that hypervascular tumor has more aggressive properties as described previously [2,16] and that this type of tumor tended to invade more deeply and invade lymphatic vessels more easily. These processes may result in lymph node metastasis in gastric cancer.

TABLE II. Microvessel Count According to Clinicopathologic Factors in Gastric Cancer Patients

Factors		Microvessel count			
		No.	Mean + SD	Median	Statistical analysis
Peritoneal metastasis	−	49	16.7 ± 9.1	15.6	N.S.
	+	6	18.7 ± 9.0	18.0	
Hepatic metastasis	−	44	15.3 ± 8.7	15.1	$P < 0.01$
	+	11	23.2 ± 8.1	28.0	
Lymph node metastasis	−	21	12.7 ± 8.5	9.4	$P < 0.005$
	+	34	19.5 ± 8.5	18.5	
Histological type[a]	pap	3	15.7 ± 11.5	10.0	N.S.
	tub	30	18.0 ± 10.2	19.0	
	por	14	15.7 ± 6.7	15.1	
	sig	5	14.2 ± 7.5	15.2	
	muc	3	17.8 ± 3.1	17.6	
Depth of tumor invasion[b]	t1	17	16.4 ± 9.4	20.0	t1 vs. t4 $P < 0.05$
	t2	23	17.1 ± 9.5	15.2	t3 vs. t4 $P < 0.005$
	t3	10	13.1 ± 6.4	11.5	
	t4	5	25.7 ± 5.7	28.0	
Macroscopic type[c]	0	19	13.7 ± 8.3	15.0	0 vs. 3 $P < 0.01$
	1	1	12.0	12.0	2 vs. 3 $P < 0.05$
	2	6	11.2 ± 3.9	10.1	
	3	20	21.2 ± 9.9	19.7	
	4	7	16.4 ± 6.2	15.6	
	5	2	25.6	25.6	
Lymphatic invasion	−	11	12.5 ± 9.3	7.0	$P < 0.05$
	+	44	18.0 ± 8.7	16.7	
Venous invasion	−	23	14.4 ± 9.2	14.8	N.S.
	+	32	18.4 ± 8.7	16.7	
Conclusive stage	Ia	13	14.4 ± 9.9	9.4	N.S.
	Ib	10	13.7 ± 6.9	12.7	
	II	8	16.8 ± 10.1	15.0	
	IIIa	2	18.5	18.5	
	IIIb	2	11.9	11.9	
	IVb	20	20.5 ± 9.0	18.5	

[a]pap, papillary adenocarcinoma; tub, tubular adenocarcinoma; por, poorly differentiated adenocarcinoma; sig, signet-ring cell carcinoma; muc, mucinous adenocarcinoma.
[b]t1, tumor invasion of mucosa or submucosa; t2, tumor invastion of muscularis propria or subserosa; t3, tumor penetration of serosa; t4, tumor invasion of adjacent structures.
[c]0, superficial, flat tumors; 1, polypoid tumors, sharply demarcated from surrounding mucosa; 2, ulcerated carcinomas with sharply demarcated and raised margins; 3, ulcerated carcinoma without definite limits, infiltrating into the surrounding wall; 4, diffusely infiltrating carcinomas; 5, nonclassifiable carcinomas. N.S. = not significant.

Microvessel count is considered to be an independent prognostic factor in numerous cancers. Tanigawa and Maeda [12,13] also demonstrated that the microvessel count was statistically significant predictor of survival in patients with gastric cancer using a multivariate analysis. We did not have enough data to analyze multivariately, but found a significant difference in survival between the hypervascular and hypovascular groups among the patients who underwent a curative operation. Curative operation means that no macroscopic tumor is left in the patient, so the presence or absence of recurrence is thought to depend mainly on tumor characteristics. There were no significant differences with patient background, including node status, depth of invasion and stage, between the two groups. Thus microvessel count appeared to be a useful independent prognostic factor in patients with gastric cancer. Four patients died of cancer in the

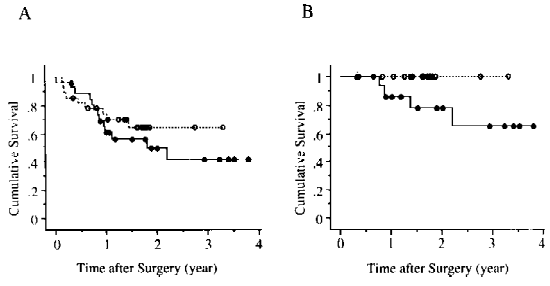

Fig. 3. Overall cumulative survival rate in all 55 patients **(A)** and in 35 patients who had undergone a curative resection **(B).** Patients were subgrouped into a hypervascular group (microvessel count ⩾ 15.6) (solid line) and a hypovascular group (microvessel count < 15.6) (dotted line). Survival of the hypervascular group was significantly worse than that of the hypovascular group in curatively resected patients ($P < 0.05$). Open and solid points represent censored cases.

hypervascular group. Two patients had hepatic metastasis and the other had peritoneal metastasis. Tanigawa et al. and Maeda et al. [12,13] reported that the microvessel density was significantly associated with the development of hematogeneous metastasis. The results obtained indicate that further study is warranted with a large cohort to confirm the association with microvessel density and hepatic metastasis after curative surgery.

Our data suggest that microvessel count is a useful predictor for metastasis and prognosis in gastric cancer. Microvessel count, in turn, is a useful tool to identify patients who need aggressive adjuvant therapy. It also suggests the possibility that inhibition of angiogenesis may prove to be of therapeutic value. A number of clinical trials using angiogenesis inhibitors are in progress [17], and microvessel count may underscore the significance of this form of therapy.

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COMMENTARY

The literature has virtually exploded with regard to angiogenesis research. Although an occasional report will state otherwise, increased new vessel ingrowth for a variety of tumors correlates with a higher propensity for dissemination and diminished patient survival. The authors' work not only adds to the plethora of evidence supporting this concept, but also adds gastric carcinoma (which has not been studied as extensively as breast and lung cancer) to the list of cancers requiring angiogenesis for growth and dissemination.

The oncologic literature should move ahead and focus less on retrospective studies of archival pathologic material. Instead, research should target angiogenesis and endothelial cell proliferation as a means of fighting cancer. Such therapy has potential advantages over conventional cytotoxic chemotherapy. First, since angiogenesis is normally required only for wound healing and menstruation, antiangiogenesis therapy should be well tolerated. Also, such therapy does not rely on a particular phase of the cell cycle to be effective. Thus far, the ideal agent has not been reported, but this area holds great promise.

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